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Development and validation of an HPLC-MS/MS method for the detection of ketamine in Calliphora vomitoria (L.) (Diptera: Calliphoridae)

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Abstract

Entomotoxicology studies the detection of drugs or other toxic substances from insects developing on the decomposing tissues. Entomotoxicology also investigates the effects of these substances on insect development, survival and morphology to provide an estimation of the minimum time since death. Ketamine is a medication mainly used for starting and maintaining anesthesia. Ketamine is also used as a recreational drug and as a sedating drug to facilitate sexual assault, resulting in several deaths. Furthermore, ketamine has been also implicated in suspicious deaths of animals. The present study describes for the first time the development and validation of an analytical method suited to detect ketamine in *Calliphora vomitoria* L. (Diptera: Calliphoridae), using liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). This study also considers the effects of ketamine on the survival, developmental rate and morphology of *C. vomitoria* immatures. Larvae were reared on substrates homogeneously spiked with ketamine concentrations consistent with those found in humans after recreational use (300 ng/mg) or allegedly indicated as capable of causing death in either humans or animals (600 ng/mg). The results demonstrated that (a) HPLC-MS/MS method is applicable to ketamine detection in *C. vomitoria* immatures, not adults; (b) the presence of ketamine at either concentration in the food substrate significantly delays the developmental time to pupal and adult instar; (d) the survival of *C. vomitoria* is negatively affected by the presence of ketamine in the substrate; (e) the length and width of larvae and pupae exposed to either ketamine concentration were significantly larger than the control samples.

Keywords: Entomotoxicology, ketamine, HPLC-MS/MS, blowflies

1. Introduction

In the process of an investigation regarding a suspicious death, toxicological analysis sometimes plays a pivotal role in identifying the presence of chemical substances that may have caused death directly (e.g. overdose) or indirectly (e.g. altering the state of awareness) (1). Historically, blood and urine represent the most commonly used biological matrices for the identification of the alleged intoxicating substances (simply referred to as “drugs” in this study) in both the living and the dead. However, over the last few years forensic toxicologists have focused their attention on the use of non-conventional biological matrices, with the aim of making the sampling less invasive and more readily available, (2). The criteria used for the selection of non-conventional matrices must be correlated with the aim of the investigation, the ease of sampling, the cost of the analyses, the reliability and reproducibility of the results, and the overall analytical complexity (2). Among the non-conventional biological matrices, the majority of studies have focused on keratine (hairs and nails), sweat, saliva, amniotic fluid and meconium (2).

The insects found on a highly decayed or skeletonized corpse can also be included as a non-conventional matrix, useful in the identification of drugs, metals, pesticides and poisons. The discipline of *entomotoxicology* involves the combination of entomology and toxicology by considering both the presence of the toxic substances in the insects that colonized the remains and their effects on the insects’ survival and development rate (3). In a forensic context, especially when the toxicological analyses have to be conducted on highly decomposed tissues, it has been demonstrated that the use of necrophagous insects provides higher sensitivity and better results compared to decomposed tissues (4, 5). Furthermore, by studying the effects of the drugs on the insects it is possible to apply appropriate correction factors to pre-existing tables of growth concerning the insects’ morphology or survival rate, and obtain a more focused estimation of the minimum time since death (minPMI) (6). Overall, entomotoxicological studies may provide information regarding both the cause and the time of death.

Ketamine, 2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone, is an arylcycloalkylamine structurally related to phencyclidine (PCP) and cyclohexamine. It was synthesized in 1962,

patented in 1963, and tested on human prisoners in 1964, with the outcome of it being a more favourable choice over PCP as a dissociative anaesthetic. After its approval in 1970, it was administered as an anaesthetic to American soldiers during the Vietnam War (7-9). At present, ketamine is a medication with unique therapeutic value in veterinary medicine, mainly used for inducing and maintaining anaesthesia (known under the name of Ketalar, Ketaminol Vet., Clorketam, Imalgene, Anesketin, Ketamine Ceva, Vetalar Vet., Narketan, Ketaset), and, to a lesser extent, it is used in human medicine especially in paediatric surgery (Ketalar, Ketamine Panpharma, Ketolar, Ketanest-S) (10). Nonmedical use of ketamine began in the 1970s, but it was not until 1999 that ketamine was introduced into the U.S. Food and Drug Administration register (11). Known also with the street name Special K, K, ket, kitkat, super k, horse trank, tac et tic, cat Valium, and vitamin K, ketamine is illegally used for its hallucinogenic effects, that cause the user to see, hear, smell, feel, and taste non-existing entities different from reality (12). Ketamine also shows dissociative effects, causing a feeling of disconnection between the mind and the body in the user ('out-of-body experience') (10). The literature reports a number of accidental/sudden death cases in which ketamine was used, alone or in combination with other drugs, e.g. cocaine, amphetamine, cannabis, or alcohol (10, 13). Ketamine has also been used in a number of drug-facilitated sexual assaults and was implicated in several deaths globally (14). To note, ketamine has also been associated with the suspicious deaths of animals (e.g. sedation with a wrong dose of the drug) and in cases of animal cruelty (15).

Within the entomotoxicological literature (16), only two studies have addressed the effects of ketamine on blowflies (17, 18). Lü *et al.* (17) investigated the effects of ketamine on *Chrysomya megacephala*'s (Fabricius) (Diptera: Calliphoridae) larval lengths, weights, and the developmental duration of larval instar, but no analytical method was developed to identify the presence of ketamine in the insects. Zou *et al.* (18) detected the presence of ketamine in *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) by gas chromatography-mass spectrometry (GC-MS), and they also observed the effects of ketamine on the development and morphology of this fly. However, the analytical method proposed by Zou *et al.* (18) did not take into account all the standard parameters suggested by the international scientific standards for validation.

The present study describes the development and validation of an analytical method based on liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), suitable to detect ketamine in immatures of *Calliphora vomitoria* L. (Diptera: Calliphoridae). Furthermore, the effects of the presence of ketamine were examined on the developmental time, the morphology (length and width) and survival of *C. vomitoria* immatures, reared on a substrate spiked with the drug.

2. Material and Methods

2.1. Preparation of foodstuff and rearing of *C. vomitoria*

Calliphorids (Diptera: Calliphoridae) are blowflies widely distributed in the different continents. Many species known to be early coloniser of dead bodies, and therefore they are used in forensic entomology for the estimation of the minPMI (20). *C. vomitoria* is distributed throughout the Holarctic region and it is mainly present in rural areas during the cold season (21-23). This fly was chosen for this study, as it is one of the most common species found in cases of forensic relevance (24).

Colonies of *C. vomitoria* were reared following the procedures described by Magni *et al.* (6, 25), starting from wild flies caught in several rural areas of the north west of Italy. Wild flies were identified by a taxonomist and regularly added to colonies to prevent inbreeding (21). As in previous research, *C. vomitoria* used in this experiment were harvested from a third generation laboratory culture. Adults were provided with distilled water and sugar *ad libitum* for their sustenance (from eclosion to the end of the experiment), while beef liver was provided as a medium for the development of oocytes (introduced on day 5 after eclosion and left 48 hours in the cage) and to obtain eggs (introduced on day 12 after eclosion) (25). The liver was checked every 2 hours and following oviposition, 3 egg clusters containing approximately 1000 eggs (1.2 g) were deposited with a fine paintbrush onto beef liver aliquots (500 g x 3) already spiked with ketamine at variable concentration levels and homogenised (control 0 ng/mg, 300 ng/mg, 600 ng/mg – simply referred as C, T1, T2 respectively). The amounts of ketamine chosen to spike the substrate were based on the concentrations found in humans after recreational use (300 ng/mg) or that which has

been indicated as capable of causing death in either adult humans (≈80 Kg) or animals of the same size (600 ng/mg) (13, 19). Liver was used as the fly food substrate because (a) it is the typical medium for forensic entomology experiments (26, 27); (b) it was used in previous research on ketamine and blowflies (18); (c) it is one of the tissues in which ketamine distributes first and its metabolic evolution starts (28). Experimental livers were homogenized with increasing volumes (0, 12.5, 25, 37.5, 50, 75, and 100 μL) of methanol solution of ketamine (1 mg/L) to reach the final concentration. Homogenization was performed using a A11 basic Analytical mill (IKA®-Werke GmbH & Co.). Following laboratory standards, a T18 digital ULTRA-TURRAX (IKA®-Werke GmbH & Co.) was used, to obtain a uniform distribution of the analytical standard. Each experimental liver was placed on a round plastic tray (Ø 14 cm with moistened paper on the base to prevent desiccation) with high sides (10 cm) to observe the start of the larvae post-feeding instar. Each plastic tray was placed on top of 5 cm of dry sand within a larger plastic box (22x40x20 cm) which was covered with a fine mesh cloth and sealed using an elastic band. Sand was used to facilitate pupation. Immature and adult flies were reared at 23.3 ± 1.2°C laboratory temperature with approximately 20% RH and a photoperiod (h) of 12:12 (L:D). Temperature data in this study were recorded using Tinytag® data-loggers with data being recorded every hour.

2.2. Sample collection

Two samples, one consisting of 30 individuals and another amounting to 1 g from each treatment were collected when *C. vomitoria* reached the second (L2), third (L3), post-feeding (PF) pupal (P) and adult (A) instars. Empty puparia (EP) were also collected.

Each sample of 30 individuals was used for morphological analyses. Specimens were sacrificed by immersion in hot water (>80°C) for 30 seconds and preserved in 70% ethanol (29). Following preservation, larvae and pupae were measured with a digital calliper (Terminator®) under a stereomicroscope (Optika SZM-2). As described by Day and Wallman (30) the length of each larva was measured between the most distal parts of the head and the eighth abdominal segment, while the width of each larva was measured between the ventral and dorsal surfaces at the junction of

the fifth and sixth abdominal segments. Regarding the pupa, the length was measured between the most distal parts, while the width was measured in the largest part of the pupal case.

Each sample weighing 1 g from each of the instars was stored at -20°C until the sampling period finished and then they were analysed to detect ketamine. Larvae of L2 and L3 instars were sacrificed and stored only after careful cleaning of each individual with water and neutral soap to remove any external contamination. Adults were not provided with any food or water source and were sacrificed 2 days after their emergence. The analytical method was validated using 50 mg of control EP, chosen as the target matrix because of their high chitin content. Empty puparia were also chosen because they can be found at the scene for a much longer period after emergence, and in such circumstances they may represent the only reliable sample for toxicological analyses (31).

When the larvae reached the PF instar, 100 individuals from each treatment were placed in separate boxes. The time to pupation, the total number of pupated individuals, as well as the time to eclosion and the total number of emerging adults were recorded.

2.3 Toxicological analysis

Chemicals and reagents – Liquid ketamine (≥99%) and d4-ketamine 100 µg/mL in methanol (as free base) ampule of 1 mL, certified reference material Cerilliant® were purchased from Sigma Aldrich® (Milano, Italy). Standard solutions of ketamine in CH_3OH (0.5 mg/L, 1 mg/L, 10 mg/L, 100 mg/L, 1000 mg/L) and d4-ketamine (used as the internal standard, ISTD) in CH_3OH (10 mg/L and 1 mg/L) were prepared from the pure liquid standards. Dichloromethane (CH_2Cl_2), methanol, trifluoroacetic acid were also purchased from Sigma Aldrich® (Milano, Italy).

Sample preparation HPLC-MS/MS analysis – Larvae (L2, L3, PF), P, EP and A samples were placed separately in falcon tubes (50 mL) and dichloromethane was added as part of the preliminary wash. The tubes with larvae and pupae were then placed in a vortex for two minutes and the organic solvent was discarded. Meanwhile, the EP were dried at room temperature under

nitrogen. Following crystallisation using liquid N_2, they were crushed with a glass rod and a 50-mg aliquot was placed in a new tube. To validate the method, control *C. vomitoria* EP were spiked with different amounts of ketamine at this stage, by adding different volumes (0, 12.5 , 25 , 37.5 , 50, 75 , and 100 μL) of methanol solution of ketamine (1 mg/L). In addition, 2 ml of CH_3OH was added and 10 mL of d4-ketamine (10 mg/L in CH_3OH) solution was added as the ISTD. The tubes were sealed and placed in heating-blocks at 60°C to extract/dissolve the matrix, for 4 hours. After elimination of the solid residues, at the digest sample was added trifluoroacetic acid (30μL) then the sample was dried at 70°C under nitrogen stream. After drying, the analytes were recovered with 200 μL of methanol. 10 μL of the solution was injected into the HPLC-MS/MS instrument.

HPLC-MS/MS analysis – Analytical determinations for the detection of ketamine was performed with LC Varian 920 coupled with Varian 320 MS operating in the electrospray ionization mode. Samples (10 μL) were injected into a Luna C18, 150mm x 2mm x 3 µm, with C18 precoloumn filter (Security Guard, Phenomenex Inc., Torrance, CA-US). Eluition mixture was composed by 87% formic acid 0.1% and 13% acetonitrile 0.1%. Temperature of drying gas was 200°C and nebulization temperature was 55°C, electron multiplier potential was 1500V. In order to complete the quantitative analysis, the mass analyzer was operated in Multiple Reaction Monitoring (MRM) and transition followed to identify ketamine were reported in Table 1.

Method validation – Ketamine detection method was validated according to the guidelines of Raposo (32), the ISO/IEC 17025 requirements and ICH guidelines (33, 34). The validation protocol included quantitative determination of ketamine in larvae, P, EP and adults: specificity, linearity, back calculation, limit of detection (LOD), limit of quantitation (LOQ), extraction recovery (ER%), repeatability, matrix effect and carry over were determined.

Specificity – Ten samples of the control EP were used to ascertain the method's specificity. Five of them were spiked with 1 mg/L of ISTD. The specificity test was successful if the S/N ratio was lower than 3 at the expected retention time of the target analytes, for all ion chromatograms.

196

197 ***Linearity –*** The linearity of the calibration model was checked by analyzing control EP samples
198 (50 mg x 5 repetitions for each calibration point) spiked with ketamine solution at concentrations of
199 0, 0.5, 0.75, 1, 1.5 and 2 ng/mg. d4-ketamine with a final concentration of 10 ng/mg was used as
200 the ISTD. The linear calibration parameters were calculated by least-squares regression, and the
201 correlation coefficient (R^2) was used for a rough estimation of the linearity. For determination of
202 linearity were considered Mandel and Olivieri's principles (35, 36). Another parameter used to
203 evaluate linearity was back calculation, which, from calibration curve point, calculates backwards
204 the concentration of ketamine in semple starting from the instrumental signal. Back calculation is
205 useful to evaluate calibration curve goodness. Quantitative results from area counts were
206 corrected using the ISTD signal.

207

208 ***Limit of detection and limit of quantitation (LOD and LOQ) –*** LOD and LOQ were calculated
209 according to Hubaux and Vos (37). This method is based on calibration curve so the result is more
210 relevant and sturdy to the method that has been developed than standard calculation of LOD and
211 LOQ.

212

213 ***Extraction recovery (ER%) –*** ER% was evaluated at two concentrations of ketamine in control EP:
214 0.75 and 2 ng/mg. For each of these concentrations, five samples were spiked before the digestion
215 step of the matrix and five after the extraction. ER% was calculated by the average ratio of the
216 analyte concentration determined after its extraction (first set) to the one determined on the spiked
217 extract (second set).

218

219 ***Repeatability (intra-assay precision) –*** Repeatability was calculated as the percent coefficient of
220 variance (CV%) after spiking ten samples of control EP with two concentrations of ketamine: 0.75
221 and 2 ng/mg. Repeatability was considered acceptable when CV% <20%.

222

Carry Over – Carry-over effect was evaluated by injecting an alternate sequence of ten blank EP semples spiked with ketamine at concentration of 0.5 ng/mg and ten blank EP samples spiked with ketamine at a concentration of 2 ng/mg. To ensure the absence of any carry-over effect, for each transition, the signal-to-noise ratio (S/N) from negative samples had to be lower than 3.

Matrix-effect – Matrix effect was evaluated following the Matuszewski's criteria (38) analysing five EP samples (chitinic matrix) spiked with ketamine at concentration of 0.25 ng/mg and five samples at 2 ng/mg both five sample of cheratin matrix at the same concentration.

2.4 Statistical analysis

Ketamine concentration in larvae, pupae and adults as well as their respective lengths and widths in different treatments were analysed by one-way ANOVA and Tukey test. Pupation and eclosion rate were analysed by a one-way ANOVA and Pearson's Chi-squared test. The level of significance was set at $P < 0.05$. Calculations were performed using IBM SPSS Statistics 22 statistical software package.

3. Results

Entomotoxicological analyses by HPLC-MS/MS confirmed the possibility that ketamine can be detected in different instars of *C. vomitoria* reared on food substrates containing ketamine in concentrations of 300 ng/mg and 600 ng/mg.

3.1 Method validation

The following parameters were obtained: coefficient of linearity (R^2), limit of detection (LOD), limit of quantitation (LOQ), extraction recovery (%), and repeatability (CV%) (Table 2). Specificity was satisfactory, while no matrix effects and carry over effects were observed.

3.2 Ketamine concentration

A summary of the ketamine concentration found in the different treatments and instars of *C. vomitoria* detected by HPLC-MS/MS is reported in Table 3.

HPLC-MS/MS analyses confirmed that the ketamine artificially added to the food substrate was present in the different immature instars of *C. vomitoria* as well as in the EP. The ketamine concentration was not found to be present in *C. vomitoria* adults analysed by HPLC-MS/MS.

The ketamine concentration was absent (lower than the LOD) in all the control samples, in the L2 of both the treatments and in all the A samples analysed by HPLC-MS/MS.

The peak of ketamine concentration was found in the L3 of both treatments and analytical methods. Overall, ketamine shows an increase in concentration until the larvae reach L3, then a decrease in the following larval instars and an increase in the P and EP. The amount of ketamine found in all treatments and instars was found to be significantly different from the controls. Statistical relevant differences were also found between T1 and T2 treatments (Table 3).

3.3 Growth rates and survival

The presence of ketamine in the food substrate had significant effects on fly development time, especially in the time from oviposition to eclosion (Table 3). The time from oviposition to pupation was similar for control larvae and for T1 larvae, but it was significantly different between control larvae and T2 larvae, that needed approximately one day more to complete pupation. The time from oviposition to eclosion was significantly different between control and larvae feeding on liver containing the two concentrations of ketamine (1-2 days more to complete metamorphosis). The difference between the treatments was not significant for either the time from oviposition to pupation and oviposition to eclosion (Table 4).

Ketamine present in the food substrate significantly affected *C. vomitoria* survival during the early instars of development (until the P instar), but it was only during metamorphosis that the effects of the presence of ketamine were extreme. Table 4 shows that during the PF instar only a maximum

of 15% of larvae died prior to pupation (2% in C; 10% in T1; 15% in T2), while during metamorphosis survival was 85% in C, 37% in T1 and 9% in T2. The survival of pupae was significantly different only between the control and both the treatments, while the survival of the adults was significantly different between all treatments.

3.4 Larval and pupal length

Significant differences were observed in the average length of larvae and pupae between the control and treatment groups (Table 5). However, significant differences occurred only in the length of advanced L3 for T2 treatment with respect to the control and T1, and in the length of P for T2 treatment with respect to the control. The length of L2, early L3 and PF of all the treatment groups were not significantly different from control (Table 5).

3.4 Larval and pupal width

Significant differences were observed in the average width of larvae and pupae between control and treatment groups (Table 6). The width of control larvae and pupae was significantly smaller than T2 individuals during the whole cycle of life. Larvae of T1 were found to have a larger width with respect to the control only in the advanced L3 stage, while during the PF instars were significantly smaller in width with respect to the T3 individuals.

4. Discussion

The use of ketamine in a medical and veterinary setting has been shown to be efficient and safe. However, in the recent past the abuse of ketamine has caused severe harm to individuals (39). A 2006 US report shows that approximately 2.3 million teens and adults have used ketamine in their lifetime (40). Ketamine it is extremely popular amongst drug users at parties all over the world and in the last 10 years the number of ketamine-related deaths have significantly increased (41). There have been major concerns in regards to driving under the influence of ketamine and the use of this

drug to facilitate sexual assault (39).

The entomotoxicology literature reports only two studies which focused on the presence of ketamine in the food substrate and its effects on blowfly development (17, 18). One study (17) considers colonies of *Ch. megacephala*, a blowfly occurring in Australasia, South Africa, Southern United States and South America (20), reared on food substrates spiked with different concentrations of ketamine. The aim of this research was to determine the effects of ketamine on blowfly development when reared at different temperatures (17). The other study (18) considers colonies of the cosmopolitan necrophagous blowfly *L. sericata*, reared on the tissue of rabbits killed following different doses of ketamine. The aims of this study were the detection of ketamine in larvae by GC-MS and the observation of the effects of ketamine on the larval morphology and development of *L. sericata* (18). The current research is the first comprehensive study regarding the effects of ketamine on *C. vomitoria* flies reared on liver homogenised with two concentrations of ketamine. The validated HPLC-MS/MS analytical procedure detected the presence of ketamine in *C. vomitoria* larvae, pupae and empty puparia. Furthermore, ketamine artificially added to the fly food substrate produces a significant increase in larval and pupal size (length and width), a significant increase in the time required to complete development and a significant decrease in the survival of this fly species especially during the period of metamorphosis.

Ketamine concentration – As stated, at present only two studies pertain to the effects of ketamine on blowflies. However, comparisons and analogies regarding the concentration of the drug in the flies can be made only with the research of Zou (18), since the other published research (17, 42) lacks any toxicological analyses of the flies reared on the food substrate spiked with ketamine.

In the research of Zou *et al*. (18) ketamine was identified by GC-MS in *L. sericata* immatures (larvae only) when reared on rabbits killed after receiving an intravenous injection of ketamine at different concentrations (1/4LD50, 1/2LD50, LD50, 2LD50). Rabbit liver and muscle containing different amounts of ketamine were used as food to rear the fly colonies. Results show that ketamine concentrations were more consistent (higher in concentration and present in several

immature instars) in treatments that had liver as food rather than muscle tissue. This is the most parsimonious explanation because (a) following GC-MS analyses of both the organs, ketamine was found to have a higher concentration in liver than in muscles and (b) liver is the organ in which ketamine metabolism occurs (28). To note, the analytical method used in the research of Zou *et al*. (18) was validated only for linearity and only 10 larvae at the different instars were used for the toxicological analysis. As well this sample consisted of 10 larvae aging from 12 to 120 hours (from L2 instar to P instar) which is not consistent in terms of analytical weight and drug content. In order to obtain reliable results, the same amount of sample should be used at the same life stage throughout the experiment.

In the present research, ketamine was identified by HPLC-MS/MS and the analytical method was validated following a set of international standards (33, 34). During the study 1 g of insect material at each instar was used as a sample for the toxicological analyses. Ketamine was detected by HPLC-MS/MS in all immature instars and pupal remains of *C. vomitoria*, Negative results in *C. vomitoria* adults were surprising, because it is known that upon emergence as an adult, the flies rapidly eliminate the drug introduced with the diet during the immature life stages (42, 43). Lastly, accordingly, to Zou *et al*. (18) ketamine was present in higher concentrations in larvae of the treatments with higher concentration of ketamine and no metabolites of ketamine were detected.

Effects of ketamine on fly growth rate and survival – *C. vomitoria* growth rate is affected by the presence of ketamine in the food substrate. In the treatment with recreational-use concentration (T1) only the time of metamorphosis was affected by the presence of the drug, while in the higher dose treatment both the period needed to reach the pupation and metamorphosis were affected. These results are in agreement with the findings regarding the effects of different ketamine concentrations on *Ch. megacephala* (17). However, they are in contrast with findings regarding *L. sericata* reared on different ketamine concentrations that showed a delay in the early development, but an overall reduction of the time needed to reach the pupal stage (18). Furthermore, *Parasarcophaga ruficornis* (Fabricius) (Diptera: Sarcophagidae) reared on different concentrations of PCP, another dissociative drug similar to ketamine, showed no significant difference in the larval

362 growth when comparing control vs treatment groups (42).
363 When considering survival data the only available information regarding ketamine and blowflies
364 demonstrates that by increasing the ketamine dosage in the food substrate the survival of *C.*
365 *vomitoria* will decrease, especially during the period of metamorphosis. A similar trend was
366 observed in *P. ruficornis* reared on different concentrations of PCP (42).
367 All previous research (17, 18, 42) underlines how similar drugs can play a role in the physiology of
368 different fly species, but before such assertions, the limitations of these studies regarding the lack
369 of repetition needs to be addressed
370
371 **Effects of ketamine on larval and pupal length and width** – Lü *et al.* (17) analysed the length of
372 *Ch. megacephala* reared on food substrates containing ketamine in doses associated with
373 1/2LD50, LD50, 2LD50 for an adult male of approximately 70 kg. It is important to note that larval
374 samples were sacrificed with a 50:50 v/v blend of ethanol and xylene and preserved in 75%
375 alcohol (17). This preservative method makes the estimation of real length difficult to compare due
376 to larval shrinkage. It is not the method recommended as a standard of best practice in forensic
377 entomology (44). Regardless of the preservation method used by Lü *et al. (*17), this research
378 showed that the relative average length of *Ch. megacephala* larvae in all the treatment colonies
379 was significant less than the control for larvae between 16 to 64 hours (= until the L3). However,
380 since the overall duration of the PF instar of the treatments was longer compared with the control,
381 the PF *Ch. megacephala* larvae in all the treatment colonies were significantly larger in length
382 compared to the control. These results, however, are not absolute measures and cannot be
383 compared with this study (47).
384 In the present research as well as in the research of Zou *et al.* (18) fly immatures are preserved
385 according to the standards and guidelines for forensic entomology, by sacrificing specimens in hot
386 water and preserving them in 70% ethanol (44). Similarly, the two studies show that larvae reared
387 on substrates enriched with ketamine are significantly longer in length when compared to the
388 respective controls (18).
389 In the present research the width of larvae and pupae was also considered. The length of fly larvae

is often used to help provide an entomological estimate of the minPMI, but the curved shape of the larvae can affect the accuracy of length measurements. The width is not affected by the curved shape of the larvae, it has been demonstrated to be comparable with body length for larval age prediction (30) and it has been used in previous entomotoxicology research (48). Despite the width measurement not often being used to measure larvae size, this data was considered in the present research as it provided a comparison with the control treatment. Statistical results on *C. vomitoria* showed that larvae and pupae reared on substrates enriched with ketamine are significantly larger in width compared to the control. As a consequence, when ketamine is present in the food substrate both width and length can be taken to estimate the age of immatures with larval width being a more accurate.

5. Conclusions

Although ketamine is important in medical and veterinary practice, it is also illegally used by humans to hallucinate and to facilitate sexual assaults. It has been a drug of choice found amongst some high profile investigations, e.g. the death of the world famous singer Amy Winehouse (2011). This research validates an analytical method based on HPLC-MS/MS to detect the presence of human recreational and lethal doses of ketamine in blowflies.

This research shows that *C. vomitoria* immature and adults accumulate ketamine and that the development and survival of *C. vomitoria* feeding on liver containing ketamine can be significantly affected by the presence of the drug.

This research underlines the need of further entomotoxicology studies, such as: a) effects of ketamine on different fly species reared at different temperature; b) the effects of “ketamine cocktails” on blowflies; c) the effects of ketamine on subsequent generations; d) the validation of alternative analytical methods (e.g. GC-MS) with the aim of allowing laboratories in possession of other analytical techniques to benefit from this type of research.

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419

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528

529 **Table 1**

530 Triple quadruple monitored transitions and applied collision energy.

531

Substance	Precursor Ion	Fragment Ion	Collision Energy (V)	Use of transition
Ketamine	238	125	20	Quantitation
	238	179	13	Identification
	238	207	10	Identification
	238	220	10	Identification
Ketamine-d4	242	129	7.5	Identification
	242	211	23.5	Identification
	242	224	10	Quantitation

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534 **Table 2**

535 Parameters calculated for ketamine using HPLC-MS/MS.

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Parameter	Value
	HPLC-MS/MS
Coefficient of linearity, R^2	0.99677
Limit of detection (ng/mg), LOD	0.015
Limit of quantitation (ng/mg), LOQ	0.031
Extraction recovery (%) at 0.75 ng/mg concentration	99.4
Extraction recovery (%) at 2 ng/mg concentration	100
CV % at 0.75 ng/mg concentration	14
CV % at 2 ng/mg concentration	16

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Table 3

Ketamine quantitation (ng/mg ± S.E.) in *C. vomitoria* (L2=second instar, L3=third instar, PF=post-feeding instar, P=pupa instar, EP=empty puparium, A=adult instar) detected through HPLC-MS/MS analysis. Quantitation was calculated using 3 replicates. Ketamine $LOD_{HPLC\text{-}MS/MS}$=0.015 ng/mg and $LOQ_{HPLC\text{-}MS/MS}$=0.031 ng/mg. The groups indicated in brackets (i.e. C, T1, T2) were significantly different (P<0.05) from the group indicated in the corresponding column.

Treatment		Control (C)	T1	T2
Amount of ketamine spiked with liver		0 ng/mg	300 ng/mg	600 ng/mg
Sampling day (Life instar)	Day 3 (L2)	<LOD	<LOD	<LOD
	Day 4 (L3)	<LOD (T1, T2)	14.9 ± 0.03 (C, T2)	180.0 ± 0.28 (C, T1)
	Day 5 (L3)	<LOD (T1, T2)	7.40 ± 0.03 (C, T2)	16.9 ± 0.24 (C, T1)
	Day 7 (PF)	<LOD (T1, T2)	0.15 ± 0.02 (C, T2)	0.97 ± 0.19 (C, T1)
	Day 9 (PF)	<LOD (T1, T2)	0.05 ± 0.02 (C, T2)	0.35 ± 0.23 (C, T1)
	Day 11 (P)	<LOD (T1, T2)	0.20 ± 0.28 (C, T2)	0.81 ± 0.11 (C, T1)
	EP	<LOD (T1, T2)	1.00 ± 0.08 (C, T2)	2.06 ± 0.21 (C, T1)
	A	<LOD	< LOD	< LOD

Table 4

Time (days ± S.E.) from oviposition to pupation and to eclosion of *C. vomitoria* larvae, which were exposed to either liver containing different amount of ketamine, or to the control liver. The table shows also the number of larvae dead prior to pupation, the number of not emerged adults, and the number of survivals. The groups indicated in brackets (i.e. C, T1, T2) were significantly different (P<0.05) from the group indicated in the corresponding column.

Treatment	Control (C)	T1	T2
Amount of ketamine spiked with liver	0 ng/mg	300 ng/mg	600 ng/mg
Larvae third instar N=	100	100	100
Time (days) from oviposition to pupation	9.89 ± 0.13 (T2)	10.30 ± 0.10	10.46 ± 0.09 (C)
Larvae dead prior to pupation	2	10	15
Pupae	98 (T1, T2)	90 (C)	85 (C)
Pupae %	98%	90%	85%
Pupae N=	98	90	85
Time (days) from oviposition to eclosion	18.40 ± 0.10 (T1, T2)	19.27 ± 0.18 (C)	20.00 ± 0.10 (C)
Not emerged adults	17	57	77
Survival	81 (T2, T3)	33 (C, T2)	8 (C, T1)
Survival %	83%	37%	9%

561 **Table 5**

562 *C. vomitoria* larvae and pupae mean lengths (mm ± S.E.) related to instar of life (L2=second instar,
563 L3=third instar, PF=post-feeding instar, P=pupa instar). The groups indicated in brackets (i.e. C, T1,
564 T2) were significantly different (P<0.05) from the group indicated in the corresponding column. For
565 each time of exposure and each treatment N=30.
566

C. vomitoria means length (mm ± S.E.)				
Treatment		Control (C)	T1	T2
Amount of ketamine spiked with liver		0 ng/mg	300 ng/mg	600 ng/mg
Sampling day (Life instar)	Day 3 (L2)	6.56 ± 0.25	6.79 ± 0.21	7.19 ± 0.16
	Day 5 (L3)	16.87 ± 0.15	16.54 ± 0.25	17.20 ± 0.29
	Day 6 (L3)	16.79 ± 0.26 (T2)	17.25 ± 0.27 (T2)	18.50 ± 0.23 (C, T2)
	Day 7 (PF)	11.91 ± 0.24	12.59 ± 0.31	12.45 ± 0.28
	Day 8 (PF)	11.56 ± 0.21	11.57 ± 0.13	11.69 ± 0.28
	Day 11 (P)	9.27 ± 0.11 (T2)	9.49 ± 0.09	9.82 ± 0.12 (C)

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570 **Table 6**

571 *C. vomitoria* larvae and pupae mean widths (mm ± S.E.) related to instar of life (L2=second instar,
572 L3=third instar, PF=post-feeding instar, P=pupa instar). The groups indicated in brackets (i.e. C, T1,
573 T2) were significantly different (P<0.05) from the group indicated in the corresponding column. For
574 each time of exposure and each treatment N=30.
575

C. vomitoria mean width (mm ± S.E.)				
Treatment		Control (C)	T1	T2
Amount of ketamine spiked with liver		0 ng/mg	300 ng/mg	600 ng/mg
Sampling day (Life instar)	Day 3 (L2)	0.89 ± 0.04 (T2)	1.03 ± 0.02	1.05 ± 0.03 (C)
	Day 5 (L3)	2.31 ± 0.50	2.30 ± 0.06	2.37 ± 0.07
	Day 6 (L3)	2.33 ± 0.07 (T1, T2)	2.70 ± 0.06 (C)	2.86 ± 0.05 (C)
	Day 7 (PF)	2.51 ± 0.07 (T2)	2.39 ± 0.06 (T2)	2.92 ± 0.04 (C, T1)
	Day 8 (PF)	2.26 ± 0.04 (T2)	2.39 ± 0.05 (T2)	2.70 ± 0.05 (C, T1)
	Day 11 (P)	2.88 ± 0.05 (T2)	3.08 ± 0.04	3.10 ± 0.06 (C)

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